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Purification and Characterization of a *Bacillus megaterium* Disulfide Reductase Specific for Disulfides Containing Pantethine 4′,4″-Diphosphate

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An NADH-linked disulfide reductase specific for disulfides containing pantethine 4′,4″-diphosphate moieties was purified 23,000-fold to homogeneity from spores of *Bacillus megaterium*. The enzyme had a native molecular weight of 122,000 with two apparently identical subunits, contained one molecule of flavin adenine dinucleotide per subunit, and was inhibited by the vicinal dithiol reagent arsenite. The enzyme was active only on disulfides containing pantethine 4′,4″-diphosphate moieties, including pantethine 4′,4″-diphosphate, oxidized coenzyme A, and coenzyme A in disulfide linkage to acyl carrier protein. However, the K_m values for pantethine 4′,4″-diphosphate and oxidized coenzyme A were 0.65 and 7.4 mM, respectively. The enzyme was at a low level in log-phase cells but increased up to 10-fold early in the stationary phase and had a similar specific activity in both the mother cell and the forespore compartment; the enzyme activity fell only slowly during spore germination and outgrowth. The enzyme was not detected in several eucaryotic sources and was present in at most a low level in a number of gram-negative bacteria. Surprisingly, the specific activity of this enzyme varied more than 200-fold in extracts from different *Bacillus* species, with values in *B. subtilis* being 5- to 6-fold lower and values in *B. cereus* and *B. sphaericus* being 8- and 35-fold higher, respectively, than the maximum value in *B. megaterium*. However, the high specific activity in *B. sphaericus* did not represent more enzyme protein than in *B. megaterium*. The possible function of this newly discovered enzyme is discussed.

Coenzyme A is a major low-molecular-weight thiol in various *Bacillus* species, and glutathione, a major thiol in many other bacteria, is absent (6, 16). In dormant spores of *Bacillus megaterium* and *Bacillus cereus*, the majority of the coenzyme A is in disulfide form, with a significant amount in disulfide linkage to protein (16). These disulfides are formed within the developing spore late in sporulation at approximately the same time that the pyridine nucleotide pool of the developing spore becomes completely oxidized; the disulfides are reduced to the thiol form in the first minutes of spore germination as NADH and NADPH are regenerated (15, 16). It has been suggested that the coenzyme A protein disulfides are involved in the heat resistance, metabolic dormancy, or both of dormant spores (16).

Previous work has identified an NADH-linked disulfide reductase in *B. megaterium* which is specific for disulfides containing pantethine 4′,4″-diphosphate moieties, including oxidized coenzyme A (26). The specific activity of this enzyme rises at least 10-fold early in sporulation and is highest in the spore (26). Given the potential importance of this enzyme to sporulation and germination in *Bacillus* species, and since this enzyme had not been characterized, we undertook its complete purification and characterization.

MATERIALS AND METHODS

Reagents and enzymes. Reduced coenzyme A (CoASH), NADH, 5,5′-dithiobis(2-nitrobenzoic acid), cyanoethyl phosphate, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), 2,2′-dipyridyl disulfide, *N*-ethylmaleimide (NEM), lysozyme, *Escherichia coli* β-galactosidase, beef liver catalase, and beef heart lactic dehydrogenase were purchased from the Sigma Chemical Co. Pantethine was purchased from the Chemical Dynamics Corporation, and sodium tetrathionate was purchased from Pfaltz and Bauer. Carrier-free $Na_2{}^{35}SO_4$ was obtained from New England Nuclear Corp. Yeast glutathione reductase was obtained from the Boehringer-Mannheim Corp., and sulfhydryl-agarose was obtained from the Pierce Chemical Co. *E. coli* acyl carrier protein was a gift of D. Louie (Baylor University, Houston, Texas).

The oxidized form of CoA (CoASSCoA) was prepared by tetrathionate oxidation of CoASH, as de-

scribed previously (26), and purified by chromatography on DEAE-cellulose as described by Moffatt and Khorana (14). Pantethine 4',4"-diphosphate was synthesized by phosphorylation of pantethine and was purified by DEAE-cellulose chromatography (14, 20). The mixed disulfide of CoASH and acyl carrier protein was synthesized by tetrathionate oxidation of a mixture of CoASH and acyl carrier protein. Acyl carrier protein (3 μmol) was dissolved in 2 ml of 10 mM KPO_4 (pH 6.8)–2 mM EDTA (buffer A) containing 10 mM dithiothreitol. After 30 min at 37°C, dithiothreitol was removed by passage through a column (2.5 by 36 cm) of Sephadex G-50 equilibrated with buffer A containing 0.1 M NaCl. Acyl carrier protein was detected by assay of its thiol group (see below), and appropriate fractions were pooled and mixed with a 12-fold molar excess of CoASH which had been purified on DEAE-cellulose. The mixture was lyophilized, redissolved in 2 ml of water, and oxidized by the stepwise addition of 15 μmol of sodium tetrathionate over a 30-min period, resulting in a greater than 80% decrease in thiol content. Gel filtration on Sephadex G-50 separated the mixed disulfide of acyl carrier protein and CoA from excess CoA and other oxidation products. Pooled fractions containing the mixed disulfide were lyophilized, dissolved in 1 ml of water, and passed through a Sephadex G-50 column equilibrated with 0.1 M ammonium bicarbonate (pH 7.5). Protein fractions were pooled and lyophilized to remove the ammonium bicarbonate. The final preparation contained 0.6 μmol of acyl carrier protein, as determined by protein analysis (2), 0.6 μmol of CoA, as determined by the optical density at 260 nm, 0.6 μmol of disulfide, as determined by the method of Zahler and Cleland (32), and no (<0.03 μmol) detectable thiol.

Sporulation, preparation of spores, and growth and extraction of bacteria and other organisms. The majority of the work was carried out with *B. megaterium* QMB1551 (originally obtained from H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass.). Cells of this organism were grown and sporulated at 30°C in supplemented nutrient broth (SNB), and spores were harvested, washed, and stored as previously described (18). Sporulating cells were fractionated into mother cell compartments and forespores as described previously (22). Some experiments also used the medium of Spizizen (24) containing 0.1% Casamino Acids, the sucrose salts medium of Slepecky and Foster (23), or L-broth. One experiment also used an asporogenous mutant (JV-114) of our *B. megaterium* strain. This organism was obtained from J. C. Vary (University of Illinois Medical Center, Chicago, Ill.) and is blocked in or before stage 1 of sporulation.

Other bacterial strains were grown in SNB at 30°C, unless otherwise noted. Cells from 100 ml of culture medium were harvested by centrifugation, washed once with cold 0.15 M NaCl, and disrupted by sonication with glass beads (1 g) in 4 ml of 50 mM KPO_4 (pH 7.5)–1 mM EDTA (buffer B). The disrupted cells were centrifuged (10 min; 10,000 × *g*), and the supernatant fluid was dialyzed against cold buffer B and stored frozen until assayed. Germinated and outgrowing spores were extracted and treated similarly. Dormant spores of *Clostridium bifermentans* were a gift of W. M. Waites (Agricultural Research Council, Norwich, England), and the spores were germinated for 30 min, as described previously (19), and extracted as described above. Rat liver and rat brain homogenates were prepared by homogenizing tissue with 5 volumes of cold buffer B in a Waring blender and then treating the homogenate as described above. Yeast extracts were prepared from commerically obtained *Saccharomyces cerevisiae* by disruption in a DynaMill (Impandex Inc.) with buffer B and then were treated as described above.

Assay procedures. The pyridine nucleotide-dependent reduction of disulfides was assayed as described previously by measuring either the generation of thiol groups or the oxidation of NADH (26). Unless otherwise noted, the substrate used was pantethine 4',4"-diphosphate at 0.5 mM. One unit of enzyme activity was defined as 1 nmol of disulfide cleaved (or NADH oxidized) per min.

FAD was assayed by measuring the ability of boiled enzyme to reactivate the D-amino acid oxidase apoenzyme (5, 13). Some FAD assays were carried out by measuring the fluorescence of the boiled enzyme before and after acid hydrolysis, as described previously by Koziol (8).

The Stokes radius of the purified enzyme was determined by gel filtration on a column (1 by 55 cm) of Bio-Gel A (1.5 M) equilibrated at 4°C in buffer A plus 0.1 M NaCl, with markers of β-galactosidase, catalase, and glutathione reductase (10). The sedimentation coefficient of the enzyme was determined in 5 to 20% sucrose gradients in buffer A plus 0.1 M NaCl with the markers given above (12). Sedimentation was for 11 h at 5°C and 40,000 rpm in an SW50.1 rotor. The molecular weight of the enzyme was determined from the products of the Stokes radius and sedimentation coefficient with reference to a standard curve of these values for standard proteins plotted against their molecular weights calculated from their amino acid sequences (21). The subunit molecular weight of the purified enzyme was determined by electrophoresis on acrylamide gels with sodium dodecyl sulfate by the method of Weber and Osborn (27). The markers used were phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase; identical results were obtained on gels containing 5 or 10% acrylamide.

Protein was routinely determined by the method of Bradford (2).

Analysis of low-molecular-weight thiols in *B. megaterium* cells and spores. *B. megaterium* cells and spores were labeled with ^{35}S by growth in a low-sulfur nutrient broth as previously described (1). Cells and spores were harvested as described previously (16) and extracted for 15 min at 80°C with 80% ethanol containing 25 mM KPO_4 (pH 7.5), 5 mM EDTA, and 25 mM ethanethiol (buffer C). This procedure extracts all low-molecular-weight compounds from cells and spores (16) and results in hydrolysis of acyl-thiols to the thiol form (25). The supernatant fluid from the ethanol extraction was flash evaporated, dissolved in a small volume of buffer C, and passed through a Sephadex G-25 column equilibrated with buffer C, and the excluded fraction was discarded. The included fraction was lyophilized to remove ethanethiol, and sulfhydryl compounds in the extract were purified by using sulfhydryl-agarose essentially as described previously by Brocklehurst et al. (3), but using 50 mM ethanethiol to elute covalently bound thiols. This procedure gave 92% recovery of [^{35}S]CoASH (purified by the procedure described above plus DEAE-cellulose chromatography) that had been added to the ethanol extract at the beginning of cell extraction. ^{35}S-

labeled thiols were separated by thin-layer chromatography on plastic-backed cellulose sheets with butanol-acetic acid-water (5:2:3) and ethanol–0.5 M ammonium acetate (66:34) as the solvents. The dried chromatograms were cut up and their radioactivity was counted in a scintillation counter. The identity of the only two major thiols found in *B. megaterium* (CoASH and 4-phosphopantetheine) was further established by DEAE-cellulose chromatography (14).

Purification of *B. megaterium* disulfide reductase. The initial steps in the purification of *B. megaterium* disulfide reductase were similar to those used previously (26), but with a number of modifications.

Step 1. The crude lysate was prepared from 100 g of spores, as described previously (26), but with the volumes of all solutions increased 3.3-fold. The crude lysate was treated with streptomycin sulfate (40 g dissolved in 200 ml of buffer B), and after incubation at 4°C for 1 h, the precipitate was removed by centrifugation and discarded.

Step 2. Ammonium sulfate was added to the streptomycin sulfate supernatant fluid to 60% saturation, and the precipitate was removed by centrifugation and discarded. Ammonium sulfate was then added to the supernatant fluid to 90% saturation, and the precipitate was removed by centrifugation.

Step 3. The 60 to 90% ammonium sulfate precipitate was dissolved in 10 mM KPO_4 (pH 7.4)–2 mM EDTA (buffer D) to give 10 mg of protein per ml (about 300 ml) and was made 20 μM in FAD and then heated at 60°C for 20 min and rapidly chilled. The turbid solution was centrifuged for 30 min at 27,000 × *g*, the precipitate was discarded, and the supernatant fluid was refractionated with ammonium sulfate, with the fraction precipitating between 55 and 90% saturation being saved. The precipitate was dissolved in buffer D (70 ml) and dialyzed overnight against two changes of 2 liters of buffer D.

Step 4. The dialyzed enzyme was adsorbed to a DEAE-cellulose column (5 by 12 cm) equilibrated with buffer D, washed with 1 liter of buffer D, and eluted with a linear gradient of 1 liter of buffer D and 1 liter of buffer D plus 0.25 M NaCl. Fractions of 20 ml were collected, and the single enzyme peak (26) was pooled.

Step 5. Ammonium sulfate was added to the enzyme from the DEAE column to 60% saturation, and the mixture was centrifuged. The supernatant fluid was applied to a column (1.5 by 12 cm) of octyl Sepharose CL-4B equilibrated with buffer D which was 60% saturated with ammonium sulfate at 4°C. The column was washed with 50 ml of buffer D which was 45% saturated with ammonium sulfate at 4°C, and enzyme was eluted with a linear descending gradient of 150 ml of buffer D which was 45% saturated with ammonium sulfate at 4°C and 150 ml of buffer D which was 25% saturated with ammonium sulfate at 4°C. The single enzyme peak was pooled and concentrated on a 0.5-ml column of octyl Sepharose, as previously described (26).

Step 6. The enzyme from step 5 (1.2 ml) was applied to a column (1.5 by 80 cm) of Bio-Gel A (0.5 M) equilibrated with buffer D plus 0.1 M NaCl, and 1.5-ml fractions were collected at a flow rate of 7.5 ml/h. The enzyme activity eluted with the major protein peak and was concentrated as described above to a volume of about 0.5 ml.

Electrophoretic purification and analysis (step 7). The enzyme from step 6 was further purified by preparative acrylamide gel electrophoresis on 7.5% gels under non-dissociating conditions at a running pH of 7.5 (29). The enzyme was applied to the wells of a slab gel (1.5 by 10 by 20 mm) and electrophoresed at 14°C and 40 mA until the tracking dye reached the bottom of the gel. Lanes at the edges of the gel were stained for 1 h with Coomassie blue and destained for 3 h to localize the enzyme, and then appropriate regions of the gel were cut into 5-mm slices, diced with a razor blade, immersed in 4 volumes of water, and incubated overnight at 4°C. Fragments of acrylamide were removed by brief centrifugation, and the enzyme was assayed by the thiol generation assay. The purified enzyme was further analyzed on denaturing 10% acrylamide gels by the procedure of Laemmli and Favre (9).

RESULTS

Enzyme purification. Previous work has shown that the *B. megaterium* disulfide reductase has its highest specific activity in dormant spores (26). For this reason, and because of the high protease levels in sporulating cells, the enzyme was purified from spores. Spores were first germinated and extracted with lysozyme, fractionated with ammonium sulfate, heat treated in the presence of FAD (see below), and fractionated on DEAE-cellulose, octyl Sepharose, and Bio-Gel A (0.5 M) (Table 1). This procedure resulted in an 1,800-fold purification with an 11% yield. The enzyme at this stage of purification was stable when stored frozen at ≥10 μg/ml for at least 6 months and was the enzyme used for most catalytic studies. However, the enzyme was not pure at this stage, since acrylamide gel electrophoresis under either nondenaturing or denaturing conditions gave a number of protein bands (Fig. 1a and c), but a minor band on the nondenaturing gel was identified as the disulfide reductase by assay of samples of a parallel unstained lane (Fig. 1b). Because of the large separation between the enzyme and the major contaminant on the nondenaturing gel, the enzyme was further purified by preparative acrylamide gel electrophoresis under nondenaturing conditions. This resulted in an overall purification of 22,800-fold with a 9% recovery (Table 1). The enzyme prepared in this manner gave a single major band on acrylamide gel electrophoresis under denaturing conditions (Fig. 1d), and was estimated to be at least 80% pure by quantitation of a scan of a stained gel.

Subunit structure and cofactor identity and stoichiometry. The molecular weight of the native enzyme was 122,000 (Table 2). However, the molecular weight by acrylamide gel electrophoresis under denaturing conditions was 55,000, suggesting that the enzyme is a dimer (Table 2). The dimeric structure of the enzyme was confirmed by cross-linking the native enzyme with dimethylsuberimidate (4) and analyzing the products on acrylamide gels under denaturing conditions according to Weber and

TABLE 1. Purification of disulfide reductase[a]

Step	Activity (U)	Sp act (U/mg of protein)	Recovery (%)	Purification (fold)
(1) Lysate plus streptomycin sulfate	9,496	0.79[b]	100	1
(2) First ammonium sulfate	9,070	2.72	95.5	3.5
(3) Heat treatment and second ammonium sulfate	7,740	6.50	81.5	8.3
(4) DEAE-cellulose	6,610	41.8	69.6	53
(5) Octyl Sepharose	4,050	1,010	42.6	1,290
(6) Bio-Gel A (0.5 M)	1.060	1,430	11.2	1,820
(7) Preparative acrylamide gel electrophoresis	850	17,900	9	22,800 (88)[c]

[a] Enzyme was purified from 100 g of spores as described in the text. All assays used the thiol generation assay with pantethine 4′-4″-diphosphate (0.5 mM) as substrate.

[b] This value is artifically low by about 25%, owing to the presence of the lysozyme used in the extraction procedure.

[c] The value in parentheses is the percentage of FAD recovery in going from step 6 to step 7, determined as described in the text.

Osborn (27). This procedure generated bands of 55,000 and 110,000 molecular weight (data not shown).

By analogy with other disulfide reductases, such as glutathione reductase and lipoic acid reductase, it seemed likely that the *B. megaterium* disulfide reductase would contain a flavin cofactor—probably FAD. Attempts to prepare an apoenzyme of the disulfide reductase by using procedures successful with other flavoproteins did not succeed. However, initial evidence that the enzyme contained FAD was obtained from heat inactivation studies, as FAD at low concentrations protected the enzyme whereas FMN did not (Fig. 2). The 1,800-fold purified enzyme was also slightly yellow and gave typical flavin absorption (λ_{max}, 370, 450, and 468 nm in the visible range) and fluorescence emission (λ_{max}, 525 nm) spectra (data not shown). The great majority of the flavin in the partially purified enzyme was FAD, and it was present at a level of 1.75 molecules of FAD per enzyme molecule (Table 2). Of the FAD in the partially purified enzyme, 88% was recovered in the

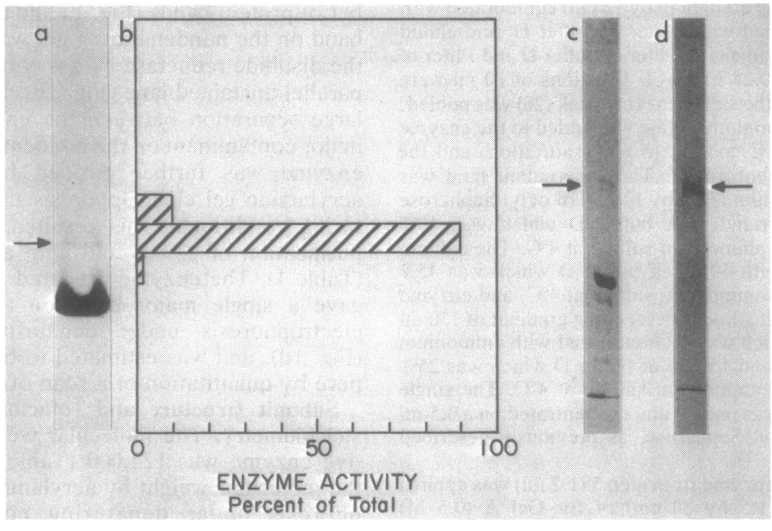

FIG. 1. Gel electrophoresis of the partially purified and purified disulfide reductase. Enzyme from step 6 (18 μg) was analyzed on two adjacent lanes of a nondenaturing slab gel as described in the text. One lane was stained (a) and the other was sliced into 5-mm strips, and enzyme was eluted and assayed (b). Enzyme from step 6 (10 μg) (c) or step 7 (1.5 μg) (d) was electrophoresed on a denaturing 10% acrylamide slab gel by the procedure of Laemmli and Favre (9) and stained and destained. The arrows note the disulfide reductase band.

TABLE 2. Molecular weight and FAD composition of disulfide reductase[a]

Property	
Native mol wt	122,000 ± 7,000
Subunit mol wt	55,000 ± 3,000
g of enzyme/1 mol of FAD (step 6 enzyme)	72,000 ± 3,000 (68,000)[b]
g of enzyme/1 mol of FAD (step 7 enzyme)	66,000 ± 3,000
mol of FAD/mol of enzyme (step 7 enzyme)	1.85[c]

[a] Native and denatured molecular weights were determined as described in the text. FAD was determined by the reconstitution of D-amino acid oxidase apoenzyme. All values are averages of at least duplicate determinations.

[b] The value in parenthesis was determined by fluorometric analysis of FAD.

[c] Calculated assuming a molecular weight for the enzyme of 122,000.

homogeneous enzyme, and enzymatic assays showed that the ratio of moles of FAD/moles of purified enzyme was 1.85 (Table 2). Thus, it seems most likely that the *B. megaterium* disulfide reductase contains two identical subunits, each with one molecule of FAD.

Catalytic properties and substrate specificity. Previous work has shown that the *B. megaterium* disulfide reductase uses NADH and only reduces disulfides containing pantethine 4',4''-diphosphate moieties (26); glutathione, lipoic acid, cystine, and pantethine are not substrates (26). CoASSCoA and pantethine 4',4''-diphosphate were the most active substrates, as both gave similar V_{max} values (Table 3). CoA in disulfide linkage to the pantetheinyl 4'-phosphoryl group of acyl carrier protein was also a substrate for the enzyme, being almost as good as CoASSCoA when both were assayed at the same concentration (Table 3). The pH optimum of the enzyme with pantethine 4',4''-diphosphate as the substrate was 6.8, and the enzyme was seven times as active in phosphate buffer as in Tris at the same pH (data not shown).

Strikingly, the K_m values for CoASSCoA and pantethine 4',4''-diphosphate were quite high (Table 3), certainly much higher than the average free concentration of these molecules in vivo. These high K_m values prompted the search for other low-molecular-weight thiols or disulfides in *B. megaterium* cells or spores. However, the only low-molecular-weight thiols that we found were CoASH, comprising about 85 to 95% of the total low-molecular-weight thiols with a total intracellular concentration (including acyl forms) of ~0.5 mM (16), and pantetheine 4'-phosphate (5 to 12% of the total). Levels of glutathione, cysteine, and pantetheine were less than 20% of the pantetheine 4'-phosphate levels (data not shown). The absence of significant levels of pantetheine in *B. megaterium* is in contrast to the recent detection of pantetheine in *B. cereus* (7).

In common with other pyridine nucleotide-linked disulfide reductases, the *B. megaterium* enzyme was inhibited by the thiol reagent NEM after preincubation with NADH (Table 4). The enzyme was also inhibited by arsenite, but again only when preincubated with NADH (Table 4), suggesting that a vicinal dithiol is involved in catalysis.

Distribution of enzyme in sporulating cells and its fate during germination and outgrowth. Previous work has shown that the level of the disulfide reductase is low in log-phase cells of *B. megaterium*, increases early in stationary-phase

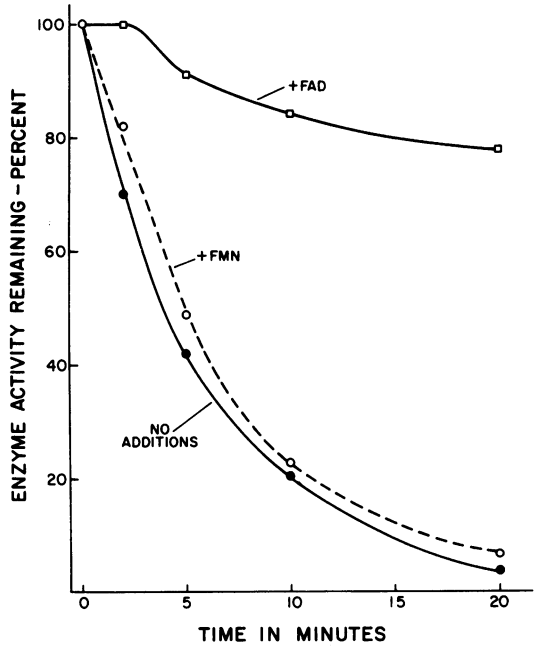

FIG. 2. Protection by FAD against heat inactivation of disulfide reductase. Enzyme was incubated in 50 mM KPO_4 (pH 6.8)–2 mM EDTA at 65°C with additions as noted; at various times aliquots (100 μl) were removed and assayed at room temperature by the NADH oxidation assay. FAD and FMN were present at 5 μM.

TABLE 3. Substrate specificity and kinetic parameters of disulfide reductase[a]

Electron acceptor	Relative activity[b]	K_m (mM)	Relative V_{max}	Turnover no. (mol/mol·min)
Pantethine 4',4"-diphosphate	100	0.67	1.0	1,300
CoASSCoA	16	7.4	1.2	1,560
Acyl carrier protein in disulfide linkage with CoA	8			

[a] Assays were carried out as described in the text with 100 μM NADH as the electron donor.
[b] Assayed at 0.5 mM electron acceptor.

growth, and is at its highest level in the dormant spore (26). However, the distribution of the enzyme was not spore specific, since it had a similar specific activity in both the mother cell compartment and the developing forespore (Table 5). The enzyme accumulated to a similar specific activity in cells grown to stationary phase in rich and poor growth media, including one (L-broth) in which there was very little sporulation. Furthermore, an asporogenous mutant of *B. megaterium* blocked early in sporulation accumulated a level of the disulfide reductase similar to that of the wild-type strain. Thus, this enzyme is neither spore nor sporulation specific.

Since the enzyme level was so low in log-phase cells (26), its fate during spore germination and outgrowth was also studied. The enzyme level fell during germination and outgrowth, but only very slowly, with a half-life ($t^{1/2}$) of >100 min, eventually reaching the specific activity found in vegetative cells (Fig. 3). This slow and incomplete loss of the disulfide reductase during germination and outgrowth is in contrast to the more rapid ($t^{1/2}$, 7 to 40 min) and complete loss of a number of spore-specific proteins during this same period (11, 17).

Panthethine 4'4"-diphosphate-specific reductase in other organisms. Since the disulfide reductase purified from *B. megaterium* is a newly discovered enzyme, its level in other organisms was determined. The enzyme was present in at most very low levels in the three eucaryotic samples analyzed, as well as in a number of gram-negative bacteria and one nonsporulating gram-positive bacterium (Table 6). Since we did not purify the low levels of activity found in these organisms, the exact nature of the enzyme

TABLE 4. Inhibition of disulfide reductase by NEM and arsenite[a]

Inhibitor (mM)	Preincubated with NADH	% Maximum activity
NEM (0.1)	Yes	7
NEM (0.1)	No	96
Arsenite (0.5)	Yes	91
Arsenite (2)	Yes	43
Arsenite (5)	Yes	12
Arsenite (5)	No	95[b]

[a] Enzyme (step 6; ~25 U) was preincubated for 10 min at 30°C with inhibitors and with or without NADH. Samples with NEM were then made 0.12 mM in 2-mercaptoethanol and, after an additional 15 min, were assayed. Samples with arsenite were assayed without 2-mercaptoethanol addition. Enzymatic activity was measured with the NADH oxidation assay.
[b] Determined from the reaction rate in the first 30 s of the assay.

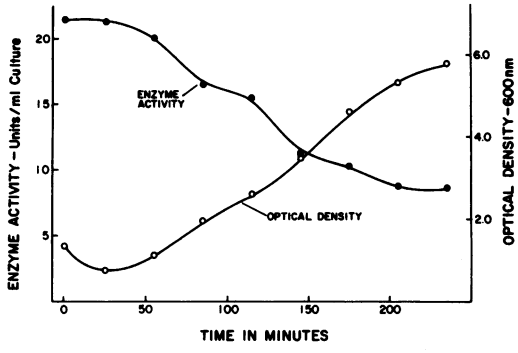

FIG. 3. Change in disulfide reductase level during germination and outgrowth. One gram of dry dormant spores in water (50 ml) was heat shocked for 30 min at 60°C and then cooled in ice. The spores were added to 200 ml of 50 mM KPO_4 (pH 7.5)–0.1 M glucose, shaken for 7 min at 30°C, centrifuged (10 min; 11,000 × *g*) and suspended in 2.9 liters of SNB. This procedure took 15 min, and >95% of the spores had initiated germination by the end of this time. The culture was shaken at 30°C, and aliquots (300 ml) were removed at various times, harvested by centrifugation (10 min; 11,000 × *g*), and washed with 40 ml of 0.15 M NaCl, and the pellets were stored frozen. Germinated and outgrowing spores were disrupted by sonication with glass beads, and extracts were treated and assayed as described in the text. The zero time point represents the dormant spore values, corrected assuming 1 g of dormant spores was 2.9 liters. The specific activity of the enzyme at 180 min was comparable to the values previously found for vegetative cells (26).

TABLE 5. Levels of disulfide reductase in cells grown in different growth media and levels in different compartments of sporulating cells[a]

B. megaterium strain	Growth medium	Sp act (U/mg of protein)		
		Whole cell	Mother cell	Forespore
Expt 1				
Wild type	SNB	0.81		
JV-114	SNB	0.72		
Wild type	Spizizen (24)	0.91		
Wild type	Slepecky and Foster (23)	0.83		
Wild type	L-broth[b]	0.83		
Expt 2				
Wild type[c]	SNB	0.97	0.60	1.22
Wild type[d]	SNB	1.01	0.66	0.75

[a] Cells were grown, harvested, fractionated, extracted, and assayed as described in the text. Unless otherwise noted, cells were harvested 4 h after the optical density stopped rising. All values are averages of at least duplicate assays.

[b] Extended incubation in this medium resulted in <1% of the cells progressing beyond stage 2 of sporulation, as determined by observation in the phase-contrast microscope.

[c] Cells were harvested 5 h after the end of log-phase growth.

[d] Cells were harvested 7 h after the end of log-phase growth.

catalyzing the reaction is not clear. However, significant levels of the enzyme were found in a number of *Bacillus* species (Table 6). Strikingly, the maximum specific activity in different *Bacillus* species varied 200-fold, from a low in *B. subtilis* to a high in *B. sphaericus*. Appropriate experiments showed that these differences were not due to the presence of inhibitors or inactivators in different species, to widely varying K_ms for pantethine 4',4''-diphosphate, or to the presence of a high-molecular-weight enzyme complex in one of these organisms (data not shown). Indeed, when the enzymes were purified about 1,000-fold from *B. cereus* and *B. sphaericus*, they were similar to the *B. megaterium* enzyme in molecular weights and K_m values (data not shown). Strikingly, analysis of the 1,000-fold-purified *B. sphaericus* enzyme on nondenaturing gels showed that the enzyme was at most 5% pure (data not shown). Thus the 35-fold-higher specific activity of the enzyme in *B. sphaericus* extracts (Table 6) compared with the specific activity of the *B. megaterium* enzyme does not represent 35-fold more enzyme protein.

DISCUSSION

The disulfide reductase described in this communication is similar to other pyridine nucleotide-linked disulfide reductases such as asparagusate dehydrogenase (31), glutathione reductase (28), lipoic acid reductase (28), and thioredoxin reductase (28), all of which use a reduced pyridine nucleotide (NADH or NADPH) to reduce a disulfide bond. All contain an FAD cofactor and, in so far as is known, contain two identical subunits (molecular weights, 35,000 to 60,000), each with an FAD. The turnover number of the *B. megaterium* enzyme with CoASSCoA is 20-fold below that of the most active disulfide reductase (glutathione reductase) but only 2.5-fold below that of thioredoxin reductase (28). Presumably, the turnover number of the *B. sphaericus* enzyme is greater than that for glutathione reductase. Although the specificity for disulfides differs among these enzymes, all seem to act by a reaction mechanism not only involving oxidation and reduction of FAD, but also involving the intermediate reduction of an enzyme cystine residue to a vicinal pair of cysteine residues (28). The inhibition of the *B. megaterium* enzyme by arsenite only after preincubation with NADH suggests that this enzyme has a similar reaction mechanism. Thus, the *Bacillus* disulfide reductase specific for disulfides containing pantethine 4',4''-diphosphate moieties is a new addition to this class of enzymes.

Given the specificity of the *Bacillus* species disulfide reductase, it seems likely that a major function of this enzyme in cells is to scavenge any 4-phosphopantetheine or CoASH which becomes oxidized and reduce it to the thiol form. In most organisms, this reaction may not require an enzyme, since the high level of reduced glutathione generated by NADPH and glutathione reductase can maintain CoA and its precursors in the thiol form via nonenzymatic thiol-disulfide exchange. Indeed, organisms which have glutathione have little, if any, pantethine 4',4''-reductase. However, *Bacillus* species have no detectable glutathione, and CoA appears to

TABLE 6. Specific activities of pantethine 4',4"-diphosphate reductase in various organisms[a]

Organism or tissue	Source	Sp act (U/mg of protein)			
		Whole cells	Log phase	Stationary phase	Germinated spores
Eucaryote[b]					
Rat liver		<0.10			
Rat brain		<0.2			
Yeast		<0.10			
Gram-negative bacteria					
E. coli B[b]	M. J. Osborn			0.4	
E. coli K-12[b]	M. J. Osborn		<0.1	0.4	
Salmonella typhimurium[b]	M. J. Osborn		<0.1	0.2	
Serratia rubidea[b]	R. Tilton			0.1	
Enterobacter cloacae[b]	R. Tilton		<0.2	0.4	
Klebsiella pneumoniae	R. Tilton			0.3	
Pseudomonas aeruginosa[b]	R. Tilton		<0.2	0.1	
Gram-positive, nonsporulating bacteria (*Staphylococcus aureus*)[b]	R. Tilton R. Tilton		<0.2	0.5	
Gram-positive, sporulating bacteria					
B. subtilis 168[c]	D. J. Tipper		0.2	0.35	
B. brevis[d]	R. P. Singh		0.3	1.0	
B. licheniformis A5	R. Bernlohr		0.51	0.8	
B. megaterium QMB1551[c]			0.2	1.3	
B. thuringiensis Kurastaki	L. Bulla		1.4	1.9	
B. cereus T[c]	H. O. Halvorson		3.8	10.3	
B. sphaericus 9602	Y. Imae		38.5	46.9	
C. bifermentans					0.9

[a] Bacteria were grown in SNB unless otherwise noted, harvested in mid-log phase (optical density at 600 nm = ~1.0) or stationary phase (3 to 4 h after the end of log phase), washed, broken, and assayed under standard conditions, as described in the text. All values are averages of at least duplicate assays.

[b] Representatives of a species known to contain glutathione (2).

[c] A strain known to lack glutathione (1, 2).

[d] Cells were grown in L-broth.

be the major low-molecular-weight thiol. In log-phase *B. megaterium* in which 50% of the CoA is in the thiol form, the total CoASH concentration is 0.24 mM, whereas in stationary phase it is 0.4 mM (16). No other low-molecular-weight thiol is present at levels greater than 10% of the level of CoASH. Thus, *Bacillus* species may require a disulfide reductase specific for CoA and its precursors.

The specific activity of this enzyme was at its highest level in stationary-phase growth of *Bacillus* species, although the amount of increase over log-phase cells varied. Although the exact reason(s) for the variation in specific activity with growth stage is not clear, it could reflect the generally higher level of oxidative metabolism in stationary-phase growth of *Bacillus* species. Even more unclear is the reason for the huge difference in specific activity of the disulfide reductase among different *Bacillus* species. This difference seems to reflect a difference in the V_{max} of the enzymes from different species, but why this should be the case is not clear.

One argument against the major function of the *Bacillus* disulfide reductase being reduction of oxidized CoA or pantethine 4'-4"-diphosphate in vivo is the high K_m values for these substrates in vitro, K_m values undoubtedly much higher than free concentrations in vivo. Possibly, we have not yet identified the true substrate in vivo. Thus, the enzyme may work on substrates which are compartmentalized or bound to another enzyme either noncovalently or covalently (as is the case with lipoic acid reductase). It is also possible that we have not yet discovered conditions under which the enzyme has a low K_m for its disulfide substrates. Interestingly, asparagusate dehydrogenase has an extremely high K_m

(20 mM) for its substrate in vitro, and this value is undoubtedly higher than the average concentration of asparagusic acid in vivo (30, 31). Since we have found no substrate for the *Bacillus* disulfide reductase more active than CoASSCoA or its precursors, at present reduction of these substrates is the only role we can ascribe to this enzyme in growing cells.

However, it seems likely that the enzyme must also be involved in the metabolism of the oxidized forms of CoA generated in developing spores and cleaved in spore germination. Thus, as NADH disappears from the developing forespore (15), the disulfide reductase can catalyze the formation of CoASSCoA (R. D. Swerdlow and P. Setlow, unpublished data). We do not know if the enzyme can also form CoA into disulfide linkages to protein (16), since the nature of the proteins involved (i.e., whether they contain a covalently bound pantetheinyl-4-phosphate) is not clear. However, CoA protein disulfides could be formed non-enzymatically via thiol disulfide exchange with CoASSCoA. In spore germination, when NADH is rapidly regenerated, the enzyme can rapidly reduce CoASSCoA as well as CoA in disulfide linkage to protein—the latter either directly or indirectly. Indeed, calculations have shown that the amount of enzyme in *B. megaterium* spores is sufficient to reduce all of the CoA disulfides of the spores within 7 min even given the high K_m of the enzyme; reduction of these disulfides during spore germination takes 5 to 10 min (16). However, it is still unclear if the formation of these spore disulfides late in sporulation plays any significant role in dormant spore physiology, or if it is simply a manifestation of the presence of a novel disulfide reductase as well as the loss in NADH late in sporulation.

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